Protocol for use with NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (E6310) and NEBNext Ultra Directional RNA Library Prep Kit for Illumina (E7420)

Note: There is a formulation change to one of the components of the kit (NEB #E6318: NEBNext RNase H). The protocol and use of this component have not changed.

Symbols

	*This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.*
	*This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the type of RNA input.*
	*Colored bullets indicate the cap color of the reagent to be added*

This protocol has been optimized using Universal Human Reference Total RNA.

RNA Sample Recommendations

The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts), divalent cation chelating agents (e.g. EDTA, EGTA, citrate), or organics (e.g., phenol and ethanol).

Treat the RNA sample with DNase I to remove all traces of DNA. Remove DNase I after treatment.

Typical Yield of rRNA-depleted RNA from a Reaction

The actual yield is dependent on the quality of the input RNA, the rRNA content of the sample, and the method used to purify the rRNA-depleted RNA. Recoveries of 3%–10% of the input RNA are typical.

RNA Input

100 ng to 1 µg total RNA in up to 12 µl total volume.

Note: The NEBNext rRNA Depletion Kit can be used with as low as 10 ng total RNA, however, for RNAseq samples we recommend using total RNA inputs 100 ng–1 µg to increase library complexity and reduce sequencing duplication rates.

⚠ *Assess quality of the input RNA by running input RNA on a bioanalyzer to determine the RIN number (RIN). Highly degraded samples (RIN #1–2) (FFPE) or partially degraded samples (RIN #2–7) will require different fragmentation times (Section 2.5).*

Starting Material: 100 ng-1µl total RNA in a 12 µl total volume.

2.1 Hybridize the Probes to the RNA

1. Prepare a RNA/Probe master mix as follows:
   NEBNext rRNA Depletion Solution 1 µl
   Probe Hybridization Buffer 2 µl

   Total Volume 3 µl
2. Add 3 µl of the above mix to 12 µl total RNA sample.
3. Mix by pipetting up and down.
4. Spin down briefly in a tabletop centrifuge, and immediately proceed to the next step.
5. Place samples in a thermocycler, and run the following program with the heated lid set at 105°C. This will take approximately 15–20 minutes to complete:
   2 minutes at 95°C
   0.1°C/sec at 95-22°C
   5 minutes hold at 22°C
6. Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step.

2.2 RNase H Digestion

1. On ice, prepare a master mix according to the following, and mix by pipetting up and down; use immediately.
   NEBNext RNase H 2 µl
   RNase H Reaction Buffer 2 µl
   Nuclease-free Water 1 µl

   Total Volume 5 µl
2. Add 5 µl of the above mix to the RNA sample from Step 6 in Section 2.1.
3. Mix by pipetting up and down.
4. Place samples in a thermocycler (with lid at 40°C) and incubate at 37°C for 30 minutes.
5. Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step to prevent non-specific degradation of RNA.

2.3 DNase I Digestion

1. On ice, prepare a DNase I Digestion Master Mix according to the following table, and mix by pipetting up and down; use immediately
   DNase I Reaction Buffer 5 µl
   DNase I (RNase-free) 2.5 µl
   Nuclease-free Water 22.5 µl

Total volume 30 µl

2. Add 30 µl of the above mix to the RNA sample from Step 5 in Section 2.2, and mix by pipetting up and down.
3. Place samples in a thermocycler (with lid at 40°C) and incubate at 37°C for 30 minutes.
4. Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step.

2.4 RNA Purification after rRNA Depletion Using Agencourt RNAClean XP

1. Add 2.2X (110 µl) Agencourt RNAClean XP Beads to the RNA sample from the previous section (Section 2.3, Step 4) and mix by pipetting up and down.
2. Incubate samples on ice for 15 minutes.
3. Place the tube on an appropriate magnetic rack to separate beads from the supernatant.
4. When the solution is clear (about 5 minutes), discard the supernatant.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once for a total of 2 washes.
7. Briefly spin the tube, and put the tube back in the magnetic rack.
8. Completely remove the residual ethanol, and air dry beads.
9. Remove the tube from the magnetic rack. Elute RNA from the beads with 8 µl nuclease-free water.
10. Mix well by pipetting up and down, and put the tube in the magnetic rack until the solution is clear.
11. Transfer 6 µl of the supernatant to a clean PCR tube.
12. Place the sample on ice and proceed to Section 2.5.

2.5 RNA Fragmentation, Priming and First Strand cDNA Synthesis

⚠ **RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 2.1. Follow protocol in 2.5A to set up the reaction. For highly degraded RNA (FFPE Samples) which do not require fragmentation proceed to Step 2.5B.**

2.5A RNA Fragmentation and Priming Starting from intact or Partially Degraded RNA:

1. Set up the following reaction and mix by gentle pipetting:
   Ribosomal depleted RNA 5 µl
   - (pink) NEBNext First Strand Synthesis Reaction Buffer (5X) 4 µl
   - (pink) Random Primers 1 µl

   Final volume 10 µl
2. ⚠ Incubate the sample at 94°C following the recomendations in Table 2.1 for fragments sizes ~200 nt.

Table 2.1. Suggested fragmentation times based on RIN number of RNA input.

RNA TYPE	RIN	FRAG. TIME
Intact RNA	>7	15 min. at 94°C
Partially Degraded RNA	2–6	7–8 min. at 94°C

Refer to Appendix A for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in

Appendix A only apply for intact RNA.

3. Transfer the tube to ice.

   First Strand cDNA Synthesis

   Dilute Actinomycin D stock solution (5 µg/µl) to 0.1 µg/µl in nuclease free water for immediate use.
   Note: Dilute solutions of Actinomycin D are very sensitive to light. In solution, Actinomycin D tends to adsorb to plastic and glass. For these reasons, unused dilute solutions should be discarded and not
   stored for further use. However, frozen aliquots of a concentrated stock solution (5 µg/µl) in DMSO are expected to be stable for at least a month at –20°C.
4. To the fragmented and primed mRNA from Step 3 in Section 2.5B (10 µl), add the following components and mix by gentle pipetting:

   (pink) Murine RNase Inhibitor 0.5 µl
   Actinomycin D (0.1 µg/µl) 5 µl
   (pink) ProtoScript II Reverse Transcriptase 1 µl
   Nuclease free water 3.5 µl

   Final volume 20 µl

   Note: If you are following recommendations in Appendix A, for longer RNA fragments, increase the incubation at 42°C from 15 minutes to 50 minutes in Step 5.
5. ⚠ Incubate the sample in a preheated thermal cycler (with heated lid set at 105°C) as follows:
   10 minutes at 25°C
   15 minutes at 42°C
   15 minutes at 70°C
   Hold at 4°C
6. Proceed directly to Second Strand Synthesis, Step 2.6.

2.5B Priming of Highly Degraded RNA (FFPE) which has a RIN ≤ 2 and does not Require Fragmentation:

1. Set up the following priming reaction and mix by gentle pipetting:
   Ribosome depleted RNA 5 µl
   (pink) Random Primers 1 µl

   Final volume 6 µl
2. Incubate the sample in a preheated thermal cycler as follows:
   5 minutes at 65°C, with heated lid set at 105°C. Hold at 4°C.
3. Transfer the tube directly to ice.

   First Strand cDNA Synthesis

   Dilute Actinomycin D stock solution (5 µg/µl) to 0.1 µg/µl in nuclease free water for immediate use.
   Note: Dilute solutions of Actinomycin D are very sensitive to light. In solution, Actinomycin D tends to adsorb to plastic and glass. For these reasons, unused dilute solutions should be discarded and not
   stored for further use. However, frozen aliquots of a concentrated stock solution (5 µg/µl) in DMSO are expected to be stable for at least a month at –20°C.
4. To the primed RNA in Step 3 (6 µl) add the following components and mix by gentle pipetting:
   (pink) NEBNext First Strand Synthesis Reaction Buffer (5X) 4 µl
   (pink) Murine RNase Inhibitor 0.5 µl
   Actinomycin D (0.1 µg/µl) 5 µl

(pink) ProtoScript II Reverse Transcriptase 1 µl
Nuclease free water 3.5 µl

Final volume 20 µl
Note: If you are following recommendations in Appendix A, for longer RNA fragments, increase the incubation at 42°C from 15 minutes to 50 minutes in Step 5.

5. Incubate the sample in a preheated thermal cycler (with heated lid set at 105°C) as follows:
   10 minutes at 25°C
   15 minutes at 42°C
   15 minutes at 70°C
   Hold at 4°C
6. Proceed directly to Second Strand cDNA Synthesis, Section 2.6.

2.6 Perform Second Strand cDNA Synthesis

1. Add the following reagents to the First Strand Synthesis reaction (20 µl):
   Nuclease-free water 48 µl
   (orange) Second Strand Synthesis Reaction Buffer (10X) 8 µl
   (orange) Second Strand Synthesis Enzyme Mix 4 µl

   Total volume 80 µl
2. Mix thoroughly by gentle pipetting.
3. Incubate in a thermal cycler for 1 hour at 16°C, with heated lid set at 40°C.

2.7 Purify the Double-stranded cDNA Using 1.8X Agencourt AMPure XP Beads

1. Vortex AMPure XP Beads to resuspend.
2. Add 144 µl (1.8X) of resuspended AMPure XP Beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on an appropriate magnetic rack to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Air dry the beads for 5 minutes while the tube is on the magnetic rack with lid open.
   Caution: Do not over dry the beads. This may result in lower recovery of DNA target.
8. Remove the tube from the magnet. Elute the DNA target from the beads into 60 µl 0.1X TE Buffer or 10 mM Tris-HCl pH 8.0. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.
9. Remove 55.5 µl of the supernatant and transfer to a clean nuclease free PCR tube.
   Note: If you need to stop at this point in the protocol samples can be stored at –20°C.

2.8 Perform End Prep of cDNA Library

1. Mix the following components in a sterile nuclease free tube:
   Purified double-stranded cDNA (Step 9, Section 2.7) 55.5 µl
   (green) NEBNext End Repair Reaction Buffer (10X) 6.5 µl
   (green) NEBNext End Prep Enzyme Mix 3 µl

   Total volume 65 µl
2. Incubate the sample in a thermal cycler (with the heated lid set at 75°C) as follows:
   30 minutes at 20°C
   30 minutes at 65°C
   Hold at 4°C
3. Proceed immediately to Adaptor Ligation.

2.9 Perform Adaptor Ligation

Dilute the (red) NEBNext adaptor* prior to setting up the ligation reaction.

INPUT RNA	DILUTION REQUIRED
100 ng	30 fold dilution in 10 mM Tris-HCl with 10 mM NaCl
> 100 ng up to 1 µg	10 fold dilution in 10 mM Tris-HCl with 10 mM NaCl

1. Add the following components directly to the End Prep Reaction
   (Caution: Do not pre-mix the components to prevent adaptor-dimer formation):
   End Prep Reaction 65 µl
   (red) Blunt/TA Ligase Master Mix 15 µl
   Diluted NEBNext Adaptor* 1 µl
   Nuclease-free Water 2.5 µl

   Total volume 83.5 µl
   *The adaptor is provided in NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600) Oligos for Illumina.
2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.
3. Incubate 15 minutes at 20°C in a thermal cycler.

A precipitate can form upon thawing of the NEBNext Q5 Hot Start HiFi PCR Master Mix. To ensure optimal performance, place the master mix at room temperature while purifying the ligation reaction using AMPure XP Beads. Once thawed, gently mix by inverting the tube several times.

2.10 Purify the Ligation Reaction Using AMPure XP Beads

Note: If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in Appendix A, Chapter 4.

1. Add nuclease-free water to the ligation reaction to bring the reaction volume to 100 µl. It is important to ensure the final volume is 100 µl prior to adding AMPure XP Beads.
   Note: X refers to the original sample volume of 100 µl from the above step.
2. Add 100 µl (1.0X) resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contain unwanted fragments **(Caution: do not discard the beads)**.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Briefly spin the tube, and put the tube back in the magnetic rack.
8. Completely remove the residual ethanol, and air dry beads for 5 minutes while the tube is on the magnetic rack with the lid open. **Caution: Do not over dry the beads. This may result in lower recovery of DNA target.**
9. Remove the tube from the magnet. Elute DNA target from the beads with 52 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.
10. Transfer the 50 µl supernatant to a clean PCR tube. Discard beads.
11. To the 50 µl supernatant, add 50 µl (1.0X) of the resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
12. Incubate for 5 minutes at room temperature.
13. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contains unwanted fragments **(Caution: do not discard the beads)**.
14. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
15. Repeat Step 14 once for a total of 2 washing steps.
16. Briefly spin the tube, and put the tube back in the magnetic rack.
17. Completely remove the residual ethanol, and air dry beads for 5 minutes while the tube is on the magnetic rack with the lid open. **Caution: Do not over dry the beads. This may result in lower recovery of DNA target.**
18. Remove the tube from the magnet. Elute DNA target from the beads with 19 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.
19. Without disturbing the bead pellet, transfer 17 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

2.11 PCR Enrichment of Adaptor Ligated DNA

⚠ **Follow Section 2.11A if you are using the following oligos (10 µM primer):**
NEBNext Singleplex Oligos for Illumina (NEB #E7350)
NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335)
NEBNext Multiplex Oligos for Illumina (Set 2, NEB #E7500)
NEBNext Multiplex Oligos for Illumina (Set 3, NEB #E7710)
NEBNext Multiplex Oligos for Illumina (Set 4, NEB #E7730)
NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

Follow Section 2.11B if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

2.11A PCR Library Enrichment

1. To the cDNA (17 µl) from Step 19, Section 2.10 add the following components and mix by gentle pipetting:
   - (blue) NEBNext USER Enzyme, 3 µl

(blue) NEBNext Q5 Hot Start HiFi PCR Master Mix, 25 µl
(blue) Index (X) Primer/i7 Primer*,** 2.5 µl
(blue) Universal PCR Primer/i5 Primer*, *** 2.5 µl

Total volume 50 µl

* The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions.
** For use with NEBNext Multiplex Oligos (NEB #E7335, #E7500, #E7710 or #E7730) use only one Index Primer per PCR reaction. For use with Dual Index Primers (NEB #E7600) use only one i7 Primer per reaction.
*** For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction.

2. PCR Cycling Conditions

CYCLE STEP	TEMPERATURE	TIME	CYCLES
USER Digestion	37°C	15 minutes	1
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	12–15*, **
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input. If 100 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR. However, optimization of PCR cycle number may be required to avoid overamplification.
** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the Bioanalyzer trace.

3. Proceed to Section 2.12 (Purify the PCR Reaction Using Agencourt AMPure XP Beads).

2.11B PCR Library Enrichment

1. To the cDNA (17 µl) from Step 19 Section 2.10 add the following components and mix by gentle pipetting:
(blue) NEBNext USER Enzyme 3 µl
(blue) NEBNext Q5 Hot Start HiFi PCR Master Mix 25 µl
(blue) Index/Universal Primer Mix* 5 µl

Total volume 50 µl

* The primers are provided in NEBNext Multiplex Oligos for Illumina, NEB #E6609. Refer to NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

2. PCR Cycling Conditions

CYCLE STEP	TEMPERATURE	TIME	CYCLES
USER Digestion	37°C	15 minutes	1
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	12–15*, **
Annealing/Extension	65°C	75 seconds	

Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input. If 100 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR.
** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the Bioanalyzer trace.

3. Proceed to Section 2.12 (Purify the PCR Reaction Using Agencourt AMPure XP Beads).

2.12 Purify the PCR Reaction using Agencourt AMPure XP Beads

Note: X refers to the original sample volume from the above step.

1. Vortex Agencourt AMPure XP Beads to resuspend.
2. Add 45 µl (0.9X) of resuspended Agencourt AMPure XP Beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Air dry the beads for 5 minutes while the tube is on the magnetic rack with the lid open.
   Caution: Do not over dry the beads. This may result in lower recovery of DNA target.
8. Remove the tube from the magnetic rack. Elute the DNA target from the beads into 23 µl 0.1X TE. Mix well on a vortex mixer or by pipetting up and down, quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.
9. Transfer 20 µl of the supernatant to a clean PCR tube. The library can be stored at –20°C.

2.13 Assess library quality on a Bioanalyzer (Agilent High Sensitivity Chip).

1. Dilute 2–3 µl of the library in 10 mM Tris or 0.1X TE.
2. Run 1 µl in a DNA High Sensitivity chip.
3. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.
   Note: If a peak at ~ 80 bp (primers) or 128 bp (adaptor-dimer) is shown in the Bioanalyzer traces; Bring up the sample volume (Step 9, Section 2.12) to 50 µl exactly with nuclease-free water and repeat the AMPure XP Bead clean up step (Section 2.12).

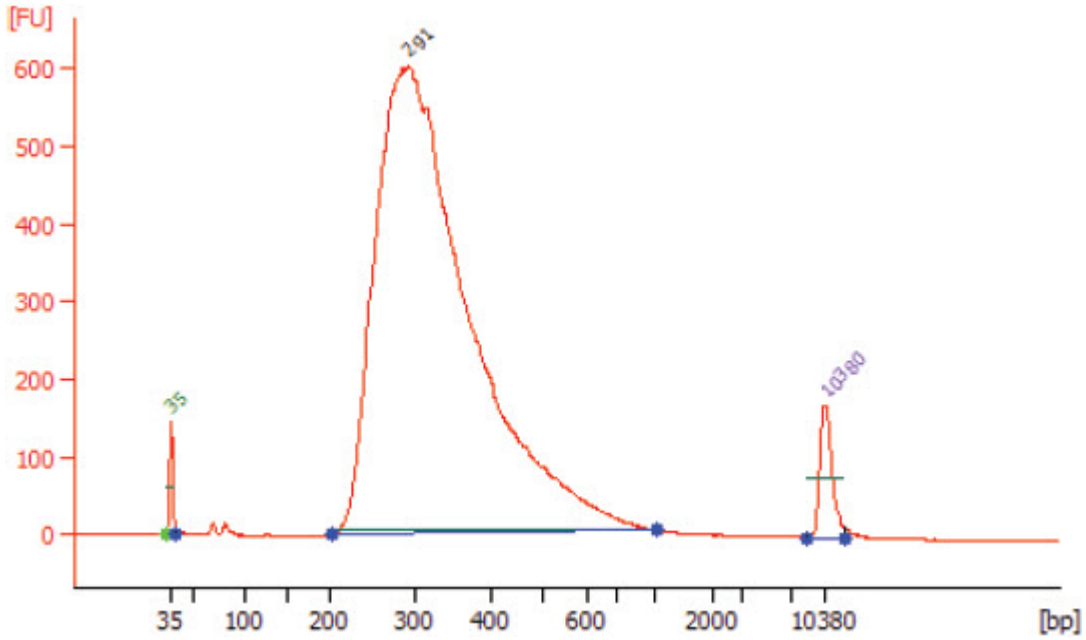

Figure 2.1: Example of RNA library size distribution on a Bioanalyzer.

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